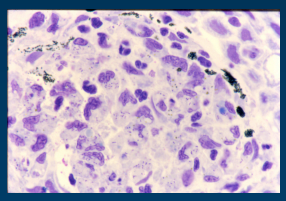
Much of the review information was taken from: Pascho RJ, Elliott DG, Chase DM. 2002. Comparison of traditional and molecular methods for detection of *Renibacterium salmoninarum*. In: Cunningham CO (ed) Molecular diagnosis of salmonid diseases. Kluwer Academic Press, Dordrecht, The Netherlands, p 157–209.

Renibacterium salmoninarum (Rs): Challenges for Diagnosis

- Two modes of transmission (vertical and horizontal)
- Chronic, intracellular existence
- Conventional diagnostic methods slow or unreliable



USGS

Topics

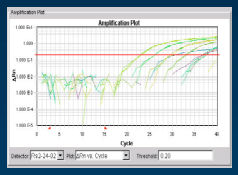
- Overview of rapid diagnostic methods used for Rs testing of fish in North America.
- Examples of comparisons among methods for Rs detection (data from testing series).

USGS

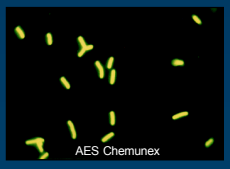
Ideal Diagnostic Test

- **Specificity**

- **Sensitivity**
- **Quantitative**



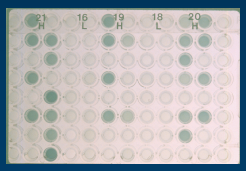
- **Rapid (≤ 2 days)**

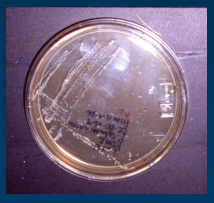


USGS

Ideal Diagnostic Test

- **Discrimination of live and dead bacteria**
- **Time and cost savings for testing multiple samples**





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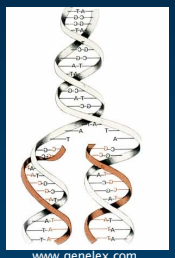
Common Rs Diagnostic Methods

- **Bacteriological culture**
- **Immunological diagnostic methods**
- **Molecular diagnostic methods**



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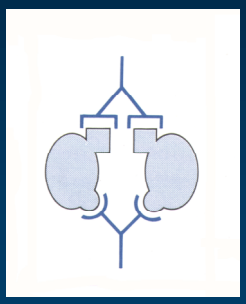
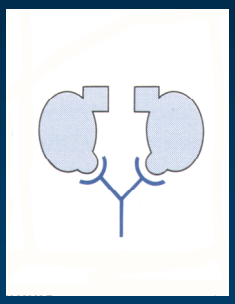
Bacteriological culture is still the gold standard by which other methods are measured even though it may take 6-19 weeks for results.

Immunological Diagnostic Methods

- **Polyclonal antibodies**
- **Monoclonal antibody**





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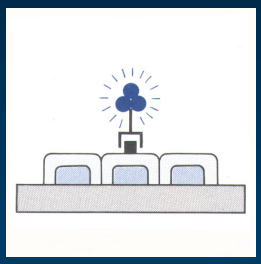
Many immunological tests developed for Rs detection, using either polyclonal antibodies (mixture of Abs with different specificities) or monoclonal antibody (single specificity).

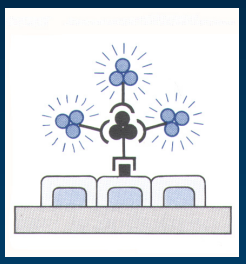
Polyclonal Ab: produced in rabbits or goats; mixture of Ab that are products of different clones of Ab-forming cells that responded to different epitopes on Ag molecule.

Monoclonal Ab: Produced by single clone of Ab-producing cells responding to a single epitope. (Fusion of desired Ab-producing cell with a mutant non-AB producing cell results in immortal hybridoma producing large quantities of Ab of a single specificity.

Fluorescent Antibody Technique (FAT)

- Direct FAT (DFAT)
- Indirect FAT (IFAT)





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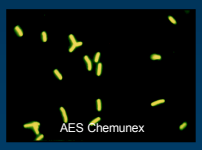
Direct FAT (DFAT): Ab conjugated directly to a fluorescent dye (usually FITC)

Indirect FAT (IFAT): Primary antibody unlabeled, secondary Ab labeled with fluorescent dye.

Fluorescent Antibody Technique (FAT)

- **Used for detection and quantification of Rs cells in smears made from homogenates of kidney tissue.**



- **Membrane filtration-FAT (MF-FAT) used for concentration and quantification of Rs in ovarian fluid samples.**

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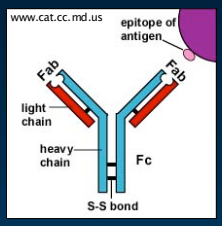
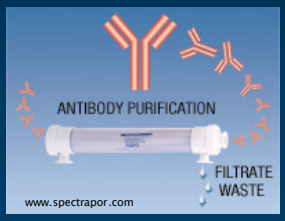
1) Standard FAT used for detection and semi-quantification of Rs in smears made from homogenates of kidney tissue or other tissues.

2) Membrane filtration-FAT (MF-FAT) specialized FAT developed for detection of Rs in ovarian fluid or water samples.

1) -- Bacteria in fluid sample first concentrated on a membrane filter by forcing fluid through filter, then bacteria on filter stained by DFAT or IFAT.

Factors Affecting FAT Specificity





- **Uniqueness of target epitopes**
- **Antibody purification procedures**
- **Number of layers in test**

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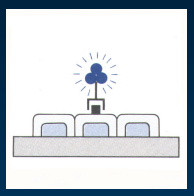
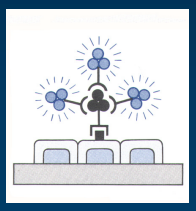
1) Specificity determined partly by uniqueness of epitopes against which monoclonal or polyclonal Ab directed,

2) and stringency of Ab purification procedures used.

3) Number of layers in FAT can affect specificity; because IFAT has more layers than DFAT, more opportunities for non-specific reactions to occur with IFAT

Factors Affecting FAT Sensitivity





- **Quality of polyclonal antiserum/monoclonal antibody**
- **Number of layers in test**
- **Appropriateness of samples (must contain whole bacteria)**

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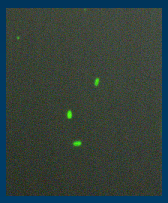
1) Quality of Ab used.

2) Number of layers: Increasing number of layers often increases no. of specific binding sites, so IFAT may yield brighter fluorescence than DFAT.

3) Samples must contain whole bacteria to yield a positive result, so must be taken from the infected tissue.

4) --(e.g. CWT study--examination of kidney smears from fish with Rs head lesions detected less than half of Rs-infected fish) Tag study: Elliott DG, Pascho RJ. 2001. Evidence that coded-wire-tagging procedures can enhance transmission of *Renibacterium salmoninarum* in Chinook salmon *Oncorhynchus tshawytscha*. J Aquat Anim Health 13:181–193.

Sensitivity of FAT



- Conventional FAT requires about 10^4 bacteria per kidney smear for a positive result.
- Membrane filtration-FAT (MF-FAT) can detect about 25-50 bacteria per ml in ovarian fluid.

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Quantification of Rs by FAT

- **Conventional smear FAT is semi-quantitative at best (qFAT more quantitative).**
- **Membrane filtration-FAT (MF-FAT) is quantitative.**
- **FAT cannot distinguish between live and dead bacteria.**

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1) Smear FAT semi-quantitative (qFAT best of these procedures).

2) Counts by MF-FAT correlate well with culture counts but are usually slightly higher because MF-FAT detects dead as well as live bacteria.

3) Current FAT cannot distinguish between live and dead bacteria.

FAT: Time and Cost


www.ion.ucl.ac.uk

- Rapid but labor-intensive; not automated.
- Equipment costs are moderate.
- Reagent and supply costs per sample:

  smear FAT ~$1.00 U.S.

  MF-FAT ~$3.00 - $6.00 U.S.

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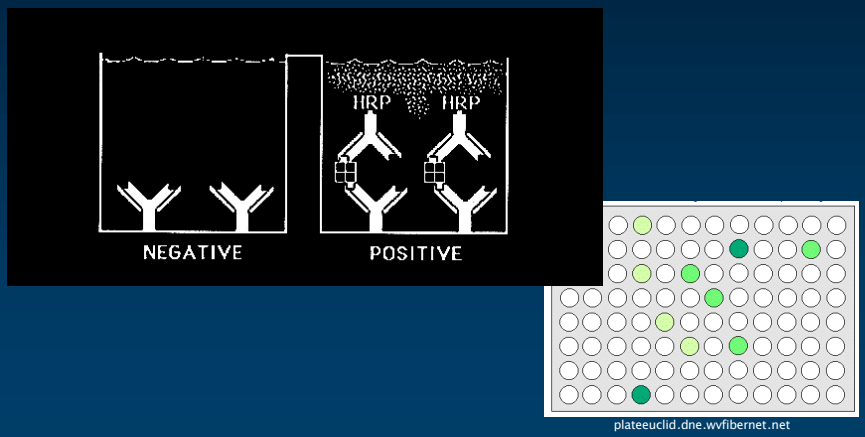
Enzyme-linked Immunosorbent Assay (ELISA)



Besides FAT, enzyme-linked immunosorbent assay (ELISA) is immunological test that has gained the widest use for Rs detection in North America.

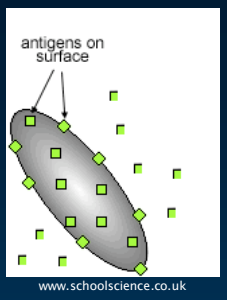
In ELISA, Rs antigen binds to an appropriate antibody that has been coated onto a solid substrate such as a tube or microtiter plate well.

After the Ag-Ab reaction, an Ab-enzyme conjugate is added, which will react specifically with any bound test Ag.

Then, the appropriate enzyme substrate is added. A positive reaction produces a color change, usually read with a spectrophotometer (although some visual assays exist).

ELISA for Rs

- **Most ELISAs use antibodies prepared against p57 protein (MSA).**
  - **Major surface-associated antigen of Rs; a virulence factor.**
  - **Produced in large amounts and released into tissues and bloodstream (soluble antigen).**



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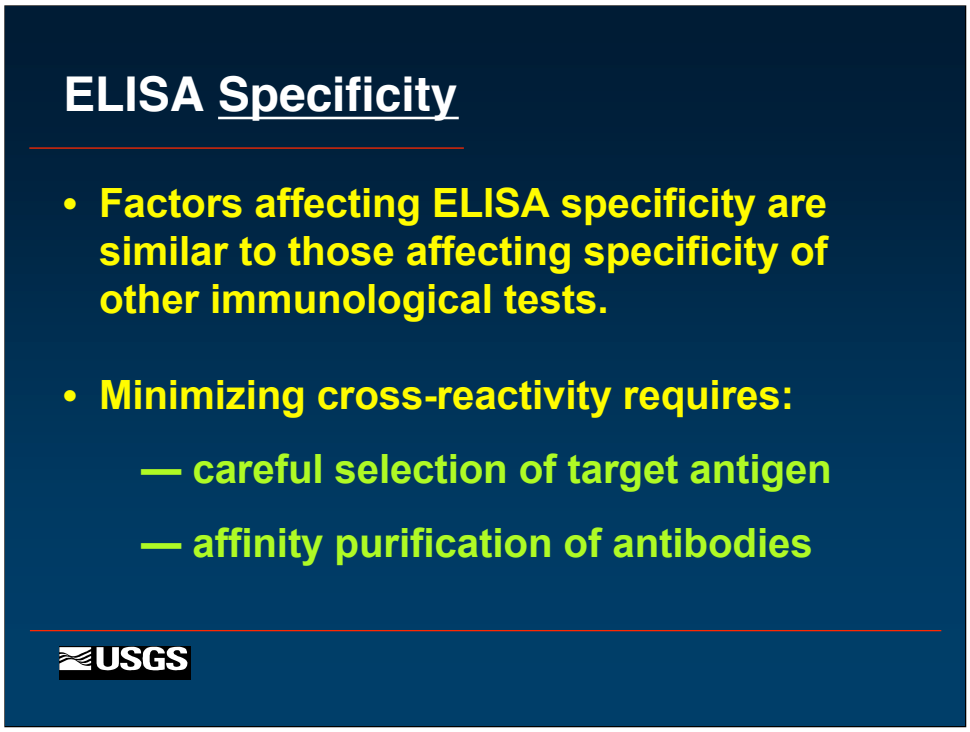
Both polyclonal and monoclonal Ab ELISAs have been developed.



1) Cross-reactivity of Ab with non-Rs bacteria has been reported with several immunological tests, including FAT and Western blots as well as ELISA.

2) Not surprisingly, cross-reactivity has been reported more often with polyclonal antisera than with Mab (but can occur with both)

3) To minimize cross-reactivity:

-careful selection of target Ag (uniqueness)

-affinity purification of Ab to select populations of Ab with desired specificities and to eliminate those with specificities to cross-reacting Ag

ELISA Sensitivity

- **ELISA is more sensitive than FAT for testing of tissue samples.**
- **Detection of p57 (MSA) concentrations as low as 3 ng per g of tissue reported for naturally infected fish.**
- **ELISA can detect circulating antigen from remote infection sites.**

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1) ELISA consistently more sensitive than FAT for testing tissue samples

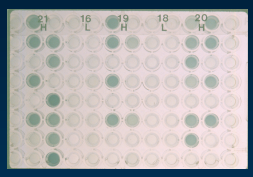
2) Detection of p57 Ag concentrations as low as 3 ng

**(but difficult to translate p57 concentration into cell numbers is difficult because the amount of p57 produced per cell may vary with the metabolic status of the bacterium in fish)

3) One advantage of ELISAs for population screening is that it can detect infections in tissues remote from the one sampled because p57 Ag originating from those sites circulates throughout the body.

Tag study: In study of Rs transmission during implantation of coded-wire tags in the snouts of fish (see photo above), fewer than half of fish tested by FAT showed positive kidney smears 4 months after tagging, but 99% were positive by ELISA testing of kidney samples, and 92% of these had high infection levels. Tag study: Elliott

Factors Affecting ELISA Sensitivity



- **Type of ELISA system used (laboratory or field test)**
- **Type of antibody used (polyclonal or monoclonal)**
- **Sample type (e.g., kidney vs ovarian fluid)**

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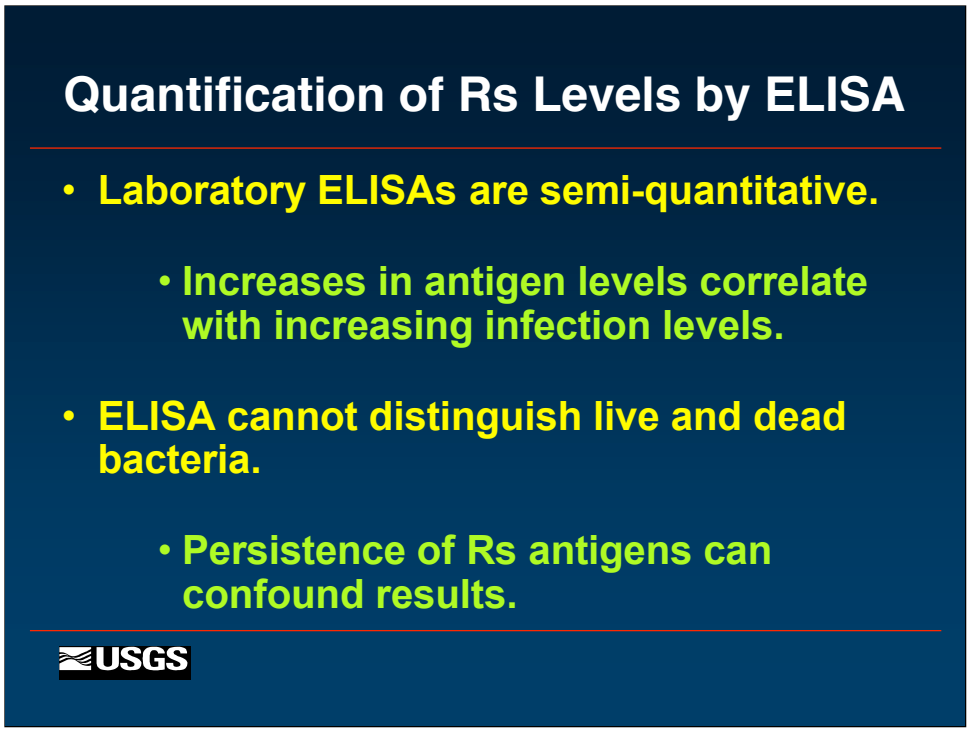
1) Lab ELISAs using spectrophotometers to read results generally more sensitive than field ELISAs using visual comparison of test samples to standards. (Field ELISAs may be mainly useful for confirming Rs in lesioned fish)

2) One study suggested higher sensitivity for polyclonal than a monoclonal ELISA for detecting Rs in fish positive by culture.

-Not surprising because polyclonal antiserum reacts with more epitopes on the p57 molecule (or other Ag) than a monoclonal Ab

3) Sensitivity also affected by sample type. Whereas ELISAs can be sensitive for detecting Rs in kidney tissue, some polyclonal and monoclonal ELISAs lack sensitivity for detecting Rs in ovarian fluid (coelomic fluid).



1)Semi-quantitative

a) Useful for monitoring changes in infection levels in a fish population

b) Field ELISAs not quantitative

2) None of ELISAs can distinguish live from dead bacteria (can be a problem for evaluating control methods such as antibiotic chemotherapy or some vaccines)

a) Persistence of Ag e.g. Vaccine study of fish injected IP with killed Rs cells with and without Ag: Rs Ag persisted for over 110 days after vaccination, and made it impossible to distinguish by ELISA the live bacteria of the challenge strain from the dead bacteria of the vaccine strain. Reference: Pascho RJ, Goodrich TD, McKibben CL. 1997. Evaluation by enzyme-linked immunosorbent assay (ELISA) of *Renibacterium salmoninarum* bacterins affected by persistence of bacterial antigens. J Aquat Anim Health 9:99–107

ELISA: Time and Cost

www.niehs.nih.gov

www-micro.msb.le.ac.uk

- Rapid; many steps automated; can test several hundred samples in one day.
- Equipment costs are negligible to high, depending on test system used.
- Reagent and supply costs per sample:

  ~$0.50 to $3.00 U.S.

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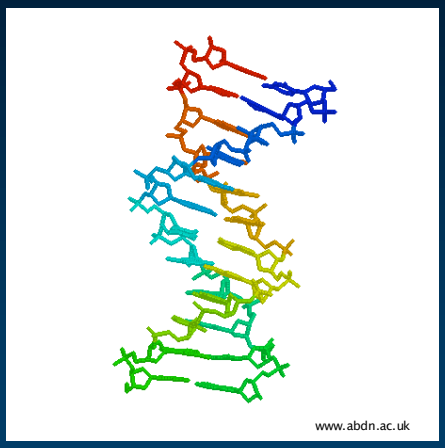
ELISA kits more expensive.

Molecular Diagnostic Tests



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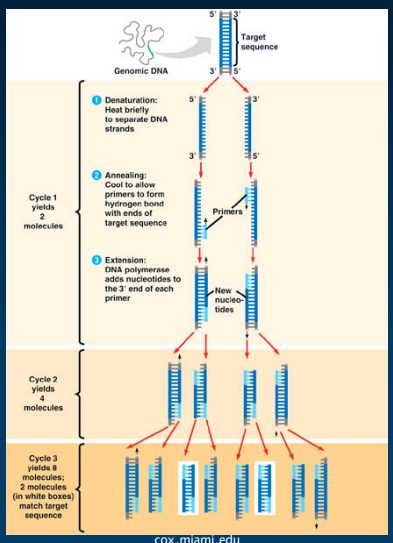
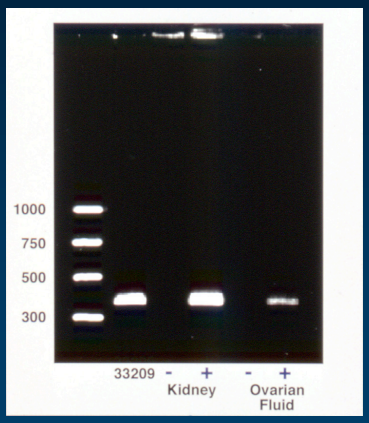
Most recent advances in Rs diagnostics: nucleic acid-based diagnostic tests.

Polymerase Chain Reaction (PCR)





Most frequently used of these tests is PCR.

Basic PCR technique based on enzymatic amplification of a specific unique DNA fragment (target DNA)

The basic PCR procedure is shown (separation of extracted DNA strands with heat,cooling and annealing of specific primers to ends of the target DNA sequence, addition of nucleotides via action of DNA polymerase to complete copying the target DNA sequence, and multiple repetition of cycles, resulting in numerous copies of the target sequence).

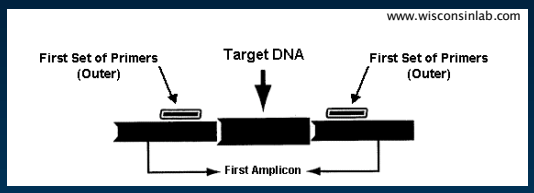
Extracted DNA sample put in a reaction tube with short synthetic single-stranded DNA primers that exactly match and flank the target DNA.

Also in reaction mixture: dinucloptide triphosphates (dNTPs; building blocks of DNA), buffers and a heat-resistant enzyme (DNA polymerase).

Heating mixture separates template strands of DNA. Upon cooling at varying temperatures, primers bind to complementary strands of target DNA, then DNA polymerase extends the bound primers in one direction, using original target DNA as a template.

End of 1 cycle: DNA count doubled. End of 30 cycles: about a billion copies of target DNA sequence.

Nested PCR



- **The first set of primers (outer) is used to amplify a sequence of nucleic acids that includes an internal target DNA sequence.**

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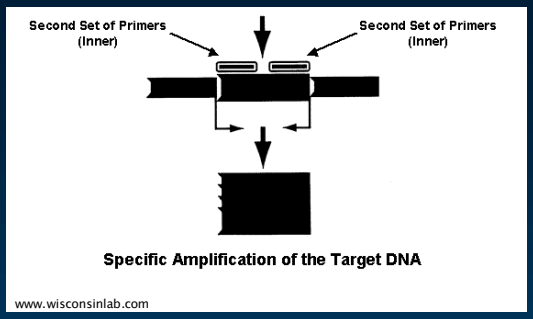
Several PCR variations have been developed:

e.g.: Improved sensitivity obtained by a 2-step or nested PCR.

1) First set of primers amplifies a sequence of nucleic acids that includes an internal target DNA or RNA sequence.
2) PCR product from first round reaction used as a template with a second set of primers that will amplify only the internal target sequence.

Besides increasing sensitivity, the internal primers used in the second round also act as an internal control by confirming the presumptive product of the initial amplification.

Nested PCR



- **The PCR product from the first round is used as a template with a second set of primers that will amplify only the inner target sequence.**

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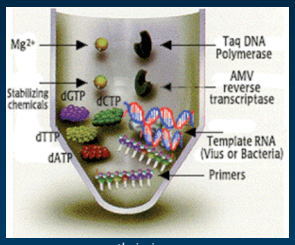
Several PCR variations have been developed:

e.g.: Improved sensitivity obtained by a 2-step or nested PCR.

1) First set of primers amplifies a sequence of nucleic acids that includes an internal target DNA or RNA sequence.
2) PCR product from first round reaction used as a template with a second set of primers that will amplify only the internal target sequence.

Besides increasing sensitivity, the internal primers used in the second round also act as an internal control by confirming the presumptive product of the initial amplification.

Reverse Transcription PCR (RT-PCR) for RNA



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- **A DNA copy complementary to the the target RNA is produced by reverse transcription.**
- **This DNA copy is then amplified by PCR.**

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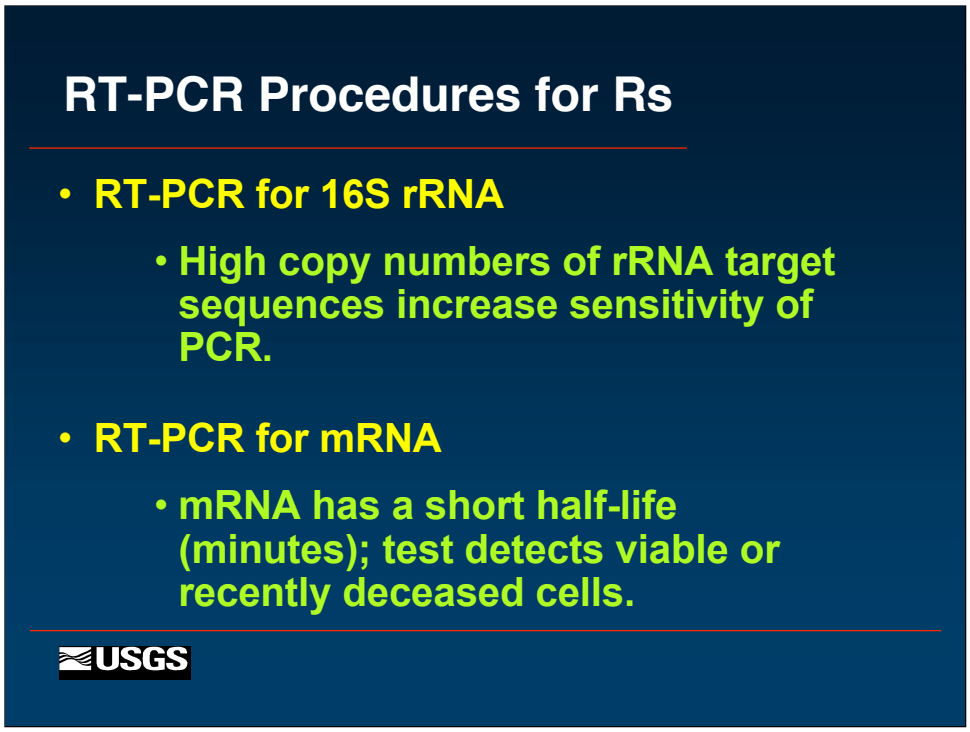
Several PCRs called reverse transcription PCR or RT-PCR developed to detect transcriptional products of a gene (RNA)

In RT-PCR, a DNA copy (cDNA) complementary to target RNA is produced enzymatically by reverse transcription.

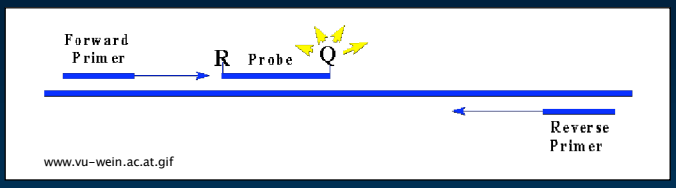
This DNA copy is then amplified by conventional or nested PCR.



RT-PCRs developed to detect specific unique sequences of the 16S subunit of ribosomal RNA

Other RT-PCR techniques have been developed to detect specific subunits of messenger RNA

Real-Time Quantitative PCR (qPCR)



- **Specific DNA probe is labeled with a reporter dye at one end and a quencher dye at the other end.**
- **When this probe is intact, the reporter dye fluorescence emission is quenched because of the physical proximity of the reporter and quencher dyes.**

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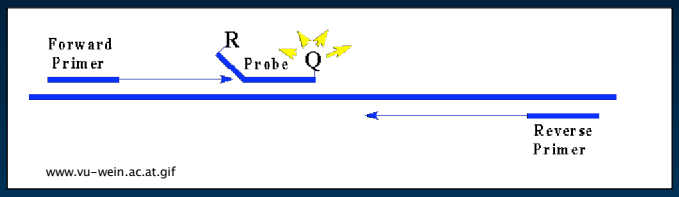
Most qPCRs for Rs: have used TaqMan system

Real-Time Quantitative PCR (qPCR)



- If the target DNA sequence is present, the probe anneals to the target and is cleaved by the nuclease activity of the *Taq* DNA polymerase as the primer extension proceeds.
- This results in the separation of the reporter dye from the quencher dye.

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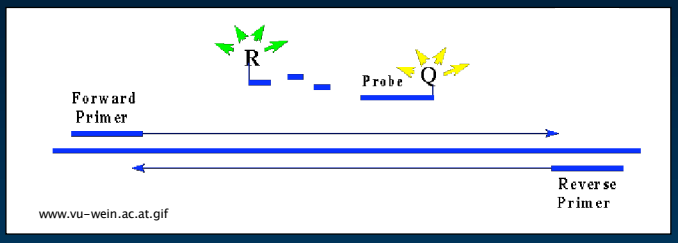
Real-Time Quantitative PCR (qPCR)



- As the separation of reporter and quencher dyes continues, the fluorescence emission increases as a function of cycle number due to increasing numbers of released reporter molecules.

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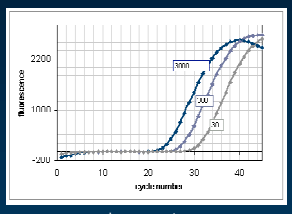
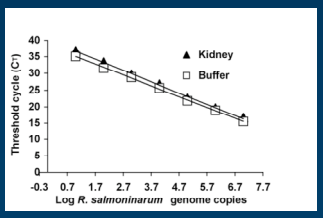
Real-Time Quantitative PCR (qPCR)

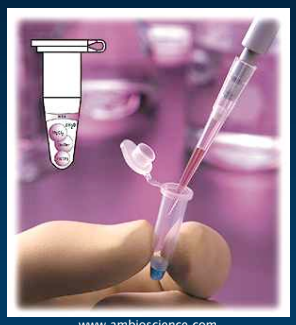


www.dxsgenotyping.com



- **The greater the initial concentration of target DNA, the lower the cycle number at which an increase in fluorescence is observed (threshold cycle).**
- **By use of a standard curve generated with a target DNA sequence of known copy number, the initial amount of DNA in unknown samples can be determined.**

Factors Affecting PCR Specificity



- **Uniqueness of target nucleic acid sequences**
- **Avoidance of contamination**

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PCRs highly specific if one has chosen a unique nucleic acid sequence for amplification.

All PCRs require a highly controlled and clean environment to avoid contamination, which can be a significant problem.

Because of the power of nucleic acid-based analysess, rigorous quality control is necessary to avoid contamination.

Any contamination of the starting sample can result in amplification of the wrong DNA sequence, causing false positive results. (Present PCRs not field tests).

Careful collection of field samples. (chlorinated-autoclaved tools, changing gloves between samples).

PCR Sensitivity

- **Detection limits: ~5 to 1000 cells per mg of sample in the presence of tissue.**
- **Some PCR assays estimated to be about 10- to 100-fold more sensitive than ELISA.**
- **Only the RT-PCR for mRNA can distinguish between live and dead bacteria.**
- **Intact target nucleic acid required.**

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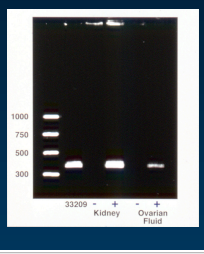
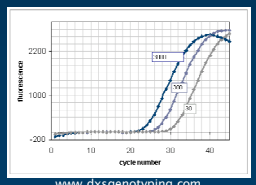
1) In our comparisons with seeded kidney tissue samples, nested PCR was about 100 times more sensitive than conventional PCR. (Sensitivity was similar when samples tested in buffer).

2) Samples must contain the target nucleic acid for a positive test. Because nucleic acid can degrade quickly outside of cells, PCRs may not detect remote infections

RNA degrades very quickly; flash frozen in liquid nitrogen mmediately or placed in a stabilizing solution (e.g. RNALater). Long-term storage at -80C. More difficult to work with in field than DNA.

Quantification of Rs Levels by PCR





www.dxsgenotyping.com

- **Most current PCR assays for Rs are not quantitative.**
- **Only the real-time quantitative PCR (qPCR) can be reliably used to monitor infection levels in fish tissues and body fluids.**

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Most current PCRs not quantitative so have limited use for monitoring changes in Rs infection levels, particularly in populations with high Rs infection prevalences such as is common in North America.

PCR: Time and Cost

PCR zonrhmapper

- **Rapid; many steps automated; sample numbers limited by capacity of equipment.**
- **Equipment costs are moderate to high (qPCR).**
- **Reagent and supply costs per sample:**

  ~$5.00 - $6.00 U.S.

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qPCR: Use of 96-well plate format increases capacity over gels.

Comparison of Rs Detection Methods: Findings from Testing Series

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The next slides show a few examples of comparisons among methods that our lab has conducted.

These illustrate some of the strengths and weaknesses of the various diagnostic tests.

We are now doing extensive testing for determination of specificity, sensitivity, and repeatability of Rs diagnostic methods.

Kidney Tissues from Spawning Chinook Salmon *Oncorhynchus tshawytscha*

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88-Fish Subsample of Root River, WI Adult Kidney Tissue, Year 2000

- Tests used:
  - Polyclonal ELISA (Kirkegaard and Perry Laboratories reagents)
  - Monoclonal ELISA (DiagXotics, Inc. kits)
    - Laboratory ELISA (K-Dtect)
    - Field ELISA (KwiK-Dtect)
  - DFAT (polyclonal Ab, Kirkegaard and Perry)
  - Nested PCR (320-bp segment of p57 gene)

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•Data from: Elliott DG, Pascho RJ. 2004. Studies on the detection, transmission and development of *Renibacterium salmoninarum infections in Great Lakes salmonid fishes. Final Report, Project 1999.51 (1999.12), Great Lakes Fishery Trust, Lansing, MI.*

•Polyclonal ELISA

•Antiserum: Polyclonal antibody, Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA

•Test: Laboratory double-antibody sandwich ELISA, 96-well microplate

•Principle: Detection of Rs antigens with polyclonal goat-anti Rs antiserum

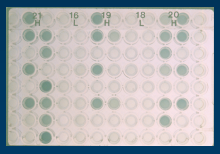
•Monoclonal ELISA

•Manufacturer: DiagXotics, Inc., Nashville, Tennessee, USA (formerly located in Wilton, Connecticut, USA)

•Test: K-Dtect laboratory ELISA, 96-well microplate

•Principle: Detection of Rs p57 protein with monoclonal antibody

88-Fish Subsample of Root River, WI Adult Kidney Tissues, Year 2000



- Fish selected on the basis of polyclonal ELISA results to provide a range of antigen levels.
- Prior to analysis by all tests, each kidney tissue sample was homogenized and distributed into separate tubes for each test.
- Samples coded for blind testing.

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•DFAT:

•Polyclonal antiserum: FITC-conjugated, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA

•Observation: Enumeration of bacteria in 100 microscope fields at 1000x magnifications

•Nested PCR:

•Primers: Designed from published sequence for gene encoding p57 protein

•First round PCR: 20 cycles

•Second round PCR: 10 cycles, using first round amplification product as template DNA

Root River, WI Kidney (88 Fish)

Test	No. Pos. of 88 fish (%)
Polyclonal ELISA	37 (42%)
Monoclonal Lab ELISA (K-Dtect)	6 (7%)
Monoclonal Field ELISA (KwiK-Dtect)	8 (9%)
DFAT	16 (18%)
Nested PCR	46 (52%)

Overall results: Highest no. positives by nested PCR, followed by polyclonal ELISA, DFAT, and the two monoclonal ELISAs.

Polyclonal ELISA Antigen Level Categories

- **Negative:** ELISA OD (absorbance) value ≤ mean OD value of negative control tissue samples + 2 SD
- **Low:** ELISA OD > negative-positive threshold value and <0.200
- **Medium:** ELISA OD 0.200 - 0.999
- **High:** ELISA OD ≥1.000

The next few slides show the distribution of positive results for each test, based on antigen level categories for polyclonal ELISA.

Root River, WI Kidney (88 Fish)

Polyclonal ELISA and Nested PCR

	Polyclonal ELISA Antigen Level Category (No. of Fish)			
	No. Fish Positive by Other Tests			
	Negative (51)	Low (11)	Medium (23)	High (3)
Nested PCR	12	9	22	3

- Nested PCR results best correlated with polyclonal ELISA.
- Difference in prevalence was largely the result of positive samples by nested PCR for fish negative by polyclonal ELISA.

Root River, WI Kidney (88 Fish)

Monoclonal ELISAs

	Polyclonal ELISA Antigen Level Category (No. of Fish)			
	No. Fish Positive by Other Tests			
	Negative (51)	Low (11)	Medium (23)	High (3)
K-Dtect	1	1	3	1
KwiK-Dtect	3	2	2	1
Nested PCR	12	9	22	3

- The monoclonal ELISAs did not detect Rs in many samples positive by both polyclonal ELISA and nested PCR.
- All samples positive by K-Dtect and KwiK-Dtect were also positive by the nested PCR, except 3 fish in the polyclonal-ELISA-negative category.

Root River, WI Kidney (88 Fish)

DFAT

	Polyclonal ELISA Antigen Level Category (No. of Fish)			
	No. Fish Positive by Other Tests			
	Negative (51)	Low (11)	Medium (23)	High (3)
DFAT	4	2	8	2

- The number of Rs cells was low (≤8 cells/100 microscope fields) in all kidney samples except two.
- The two samples with higher DFAT Rs counts had polyclonal ELISA OD values ≥0.900, suggesting medium to high Rs infection levels.

Kidney Tissues from Juvenile Chinook Salmon *Oncorhynchus tshawytscha*

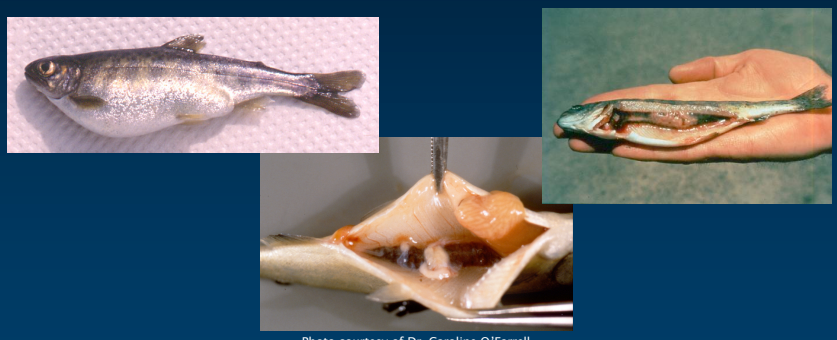

Photo courtesy of Dr. Caroline O'Farrell

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38-Fish Sample of Soos Creek, WA Yearling Chinook Salmon, Year 2005

- Tests used:
  - Polyclonal ELISA (Kirkegaard and Perry Laboratories)
  - Nested PCR
  - Real-time quantitative PCR (qPCR)

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•Manuscript including these data is in review.

•Some fish sampled showed clinical signs of BKD.

•qPCR probe and primers designed to detect specific sequences of the p57 (msa) gene. TaqMan procedure.

Soos Creek, WA Kidney (38 Fish)

Test	No. Pos. of 38 fish (%)
Polyclonal ELISA	27 (71%)
Nested PCR	25 (66%)
qPCR	27 (71%)

No difference in Rs prevalence detected by the 3 tests.

Soos Creek, WA Kidney (38 Fish)

	Polyclonal ELISA Antigen Level Category (No. of Fish)			
	No. Fish Positive by Other Tests			
	Negative (11)	Low (9)	Medium (8)	High (10)
Nested PCR	2	5	8	10
qPCR	6	3	8	10

- All samples showing medium to high antigen levels by ELISA were positive by both PCRs.
- Discrepancies were observed in samples testing negative or showing low antigen levels by ELISA.

Soos Creek, WA Kidney (38 Fish) Possible Reasons for Discrepancies

- PCR positive, ELISA negative:
  - Higher sensitivity of PCR
- ELISA positive, PCR negative:
  - Smaller sample weight for PCR
  - ELISA can detect circulating Rs antigen from remote infection sites.
  - Rs antigen can persist in the absence of live bacteria.

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•Sample weight: 300 mg for ELISA vs 40 for PCR; only about 2.5% percent of total volume of DNA extracted from each sample subjected to PCR amplification.

Ovarian Fluid from Spawning Chinook Salmon *Oncorhynchus tshawytscha*

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105 Fish, Root River, WI
Ovarian Fluid Samples, Year 1999

- Tests used:
  - Polyclonal ELISA (Kirkegaard and Perry Laboratories)
  - Membrane Filtration-FAT (MF-FAT)
  - Nested PCR

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•Data from: Elliott DG, Pascho RJ. 2004. Studies on the detection, transmission and development of *Renibacterium salmoninarum infections in Great Lakes salmonid fishes. Final Report, Project 1999.51 (1999.12), Great Lakes Fishery Trust, Lansing, MI.*

Root River, WI Ovarian Fluid (105 Fish)

Test	No. Pos. of 105 fish (%)
Polyclonal ELISA	14 (13%)
MF-FAT	34 (32%)
Nested PCR	54 (51%)

Overall results: Highest no. positives by nested PCR, followed by MF-FAT, then polyclonal ELISA

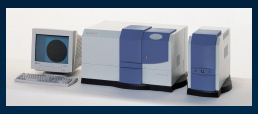
Root River, WI Ovarian Fluid (105 Fish)

	Polyclonal ELISA Antigen Level Category (No. of Fish)			
	No. Fish Positive by Other Tests			
	Negative (91)	Low (6)	Medium (6)	High (2)
MF-FAT	23	3	6	2
Nested PCR	41	5	6	2

- All samples showing medium to high antigen levels by ELISA were positive by PCR and MF-FAT.
- Differences in prevalence were largely the result of positive samples by nested PCR and MF-FAT for fish negative by ELISA.

(Nested PCR is most sensitive test among the three, but is not quantitative, so MF-FAT may sometimes be needed for quantification.)

Future Developments: Solid-Phase Laser Scanning Cytometry



AES Chemunex

- **Used for filterable samples.**
- **Taxonomic stain: ID by Immunofluorescence or fluorescence in situ hybridization.**
- **Taxonomic stain has been combined with live/dead stain.**
- **Sensitive (can detect 1 target organism on filter with 10^8 non-target organisms).**
- **Rapid: Filter scanned and mapped in 3 minutes; visual confirmation of results.**

1) QPCR is being tested for quantification of Rs in ovarian fluid, but there a lesser-known procedure that may be extremely useful, solid-phase laser scanning cytometry.

2) Solid-phase cytometry used for samples such as water or ovarian fluid. Detection ability limited only by amount of sample that can be filtered.

3) Has been used for detection of bacteria and protozoa. Can detect 1 target organism in 10^8 non-target organisms.

Comparison of Rs Detection Methods: Summary from Testing Series

- Sensitivity of nested PCR and qPCR is equal to or greater than that of polyclonal ELISA for detection of Rs in kidney tissue.
  - Some negative PCR results/positive ELISA results may be due to persistence of Rs antigen or detection of remote infections by the polyclonal ELISA.
  - DFAT is less sensitive than PCR or polyclonal ELISA, but more sensitive than the monoclonal ELISAs (K-Dtect and QwiK-Dtect).

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•Summary from testing series shown and additional series not shown.

Comparison of Rs Detection Methods: Summary from Testing Series

- Nested PCR has been the most sensitive test for screening ovarian fluid for Rs.
  - Because the nested PCR is not quantitative, MF-FAT is also useful for quantifying Rs in ovarian fluid.
  - New procedures (qPCR and solid-phase cytometry) may prove useful for quantification of Rs in ovarian fluid.

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Comparison of Rs Detection Methods: Summary from Testing Series

- Polyclonal ELISA is not a sensitive test for screening ovarian fluid for Rs.
  - The polyclonal ELISA does not consistently detect Rs antigen until MF-FAT counts exceed 10^4 or 10^5 bacteria/mL.
  - Published reports indicate that some monoclonal ELISAs are also unsuitable for screening ovarian fluid for Rs.

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•K-Dtect and KwiK-Dtect not recommended for ovarian fluid.

The Best Diagnostic Test?

Specificity	**PCR**
Sensitivity	**PCR**
Quantification	**Culture (all samples)** **MF-FAT (ovarian fluid)** **qPCR, qFAT (kidney)**
Time/cost savings for multiple samples	**ELISA** **qPCR**

The Best Diagnostic Test?

Non-lethal sample	ELISA (blood) PCR (blood, ovarian fluid, gill?) MF-FAT (ovarian fluid) Culture (ovarian fluid)
Detection of remote infections	ELISA
Detection of viable bacteria	Culture RT-PCR for mRNA

Diagnostic Methods: Other Important Factors

- Initial cost
- Specialized equipment
- Technical knowledge required
- Reagent availability

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1) Initial cost of equipment setup

2) Requirements for specialized equipment

3) Technical knowledge required (suitable for field biologists with limited training, or specialized laboratory expertise required)

4) Reagent availability: Uniform commercial source of reagents desired

5) Unreasonable expectations. Diagnosticians often looking for a “silver bullet,” I.e. one test that will serve all purposes, when it is often better to employ more than one test in critical situations or or to provide several types of information.

Diagnostic Methods: Summary

- **No single "perfect" diagnostic test exists for Rs.**
- **A diagnostic test should be selected because it is the appropriate application to answer a given question, not because it is the newest and "sexiest" test available.**
- **In certain situations, two or more tests, based on different diagnostic principles, may be needed to answer a question or confirm a result.**

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Now working on a project funded by the Great Lakes Fishery Trust for validation of non-culture Rs diagnostic methods by extensive testing of specificity, sensitivity and repeatability of immunological and molecular methods in comparison to culture.


Acknowledgements
WFRC Staff:
Tony Murray
Connie McKibben
Lynn (Chip) Applegate
Carla Conway
Maureen Purcell
Sue Marcquenski
(Wisconsin DNR)
Washington
Department of Fish
and Wildlife
Funding: Great Lakes
Fishery Trust
USGS
science for a changing world